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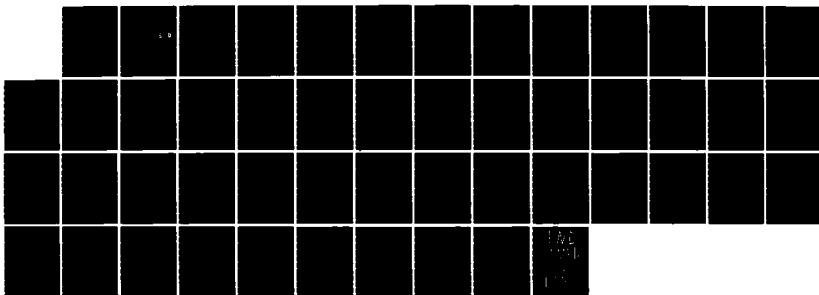
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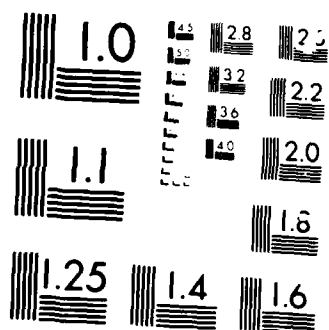
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THE SCREENING AND EVALUATION OF  
EXPERIMENTAL ANTIPARASITIC DRUGS

Annual Report

Arba L. Ager, Jr., Ph.D.

December 1983

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## ABSTRACT

The investigations undertaken during this report period included three antimalarial screens, two African trypanosome screens, and an American trypanosome screen. The three antimalarial screens were; a primary blood schizonticidal test using *Plasmodium berghei*, a primary causal prophylactic test using *Plasmodium yoelii* and *Anopheles stephensi* mosquitoes, and a secondary drug screening system using both of the above species of rodent malaria. The two African trypanosome systems included a primary screen and a secondary drug-resistant screen. The American trypanosome system was a primary test.

In the primary antimalarial blood schizonticidal test 3,026 three dose-level tests were done with 335 compounds exhibiting activity. In the prophylactic antimalarial tests 1,571 three dose-level tests were performed to detect activity against sporozoites and/or exoerythrocytic stages. 89 compounds were active. In the regular secondary test 3 compounds were tested against the drug-sensitive P-line of *P. berghei*, while, 13 compounds were tested against the drug-resistant lines. Other special tests included. 1) Determining how stable the drug-resistant lines would be when drug pressure is removed. 2) Determination if the development of resistance to mefloquine was slowed when sulfadoxine and pyrimethamine were co-administered. 3) Development of mefloquine resistance in a standardized method.

The primary test in African trypanosomiasis evaluated compounds for trypanosomicidal activity against a drug-sensitive line. 2069 three dose-level tests were performed with 82 compounds exhibiting activity. In the drug-resistant screen 10 compounds were tested for activity against one or more resistant lines. A special test was done to see how long resistance lasted in the pentamidine, melarsoprol, and suramin-resistant lines.

In the primary test in American trypanosomiasis 126 dose-level tests were performed. Three compounds were found to be active.

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## FOREWORD

In conducting the research described in this report, the Investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHHS Publication No. 85-23 (NIH), Revised 1985).

## ABSTRACT

The investigations undertaken during this report period included three antimalarial screens, two African trypanosome screens, and an American trypanosome screen. The three antimalarial screens were; a primary blood schizonticidal test using Plasmodium berghei, a primary causal prophylactic test using Plasmodium yoelli and Anopheles stephensi mosquitoes, and a secondary drug screening system using both of the above species of rodent malaria. The two African trypanosome systems included a primary screen and a secondary drug-resistant screen. The American trypanosome system was a primary test.

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The primary test in African trypanosomiasis evaluated compounds for trypanosomicidal activity against a drug-sensitive line. 2069 three dose-level tests were performed with 82 compounds exhibiting activity. In the drug-resistant screen 10 compounds were tested for activity against one or more resistant lines. A special test was done to see how long resistance lasted in the pentamidine, melarsoprol, and suramin-resistant lines.

In the primary test in American trypanosomiasis 126 dose-level tests were performed. Three compounds were found to be active.

SCREENING PROCEDURE FOR ASSESSING THE BLOOD SCHIZONTICIDAL  
ANTIMALARIAL ACTIVITY OF CANDIDATE COMPOUNDS  
IN PLASMODIUM BERGHEI INFECTED MICE

The recognition of chloroquine-resistant strains of Plasmodium falciparum in South America and Southeast Asia first posed what is now a critical problem in the chemotherapy of malaria. Parasite resistance to 4-aminoquinolines (e.g. chloroquine and amodiaquine), antifolates (e.g., pyrimethamine) and other standard antimalarial compounds such as quinine, has caused an increase in concern for the development of safe alternative therapeutic agents.

The World Health Organization currently estimates that over 100 million cases of malaria worldwide require treatment each year. Recently, chloroquine-resistant parasites have been noted in Africa where over one million children die from malaria yearly. Reports from endemic areas in the world indicate a significant resurgence of malaria. The number of cases of malaria reported from Haiti exceeded 100,000 during 1982. During 1982 there were over 1,300 cases of malaria brought into the United States. The current widespread endemicity of malaria, with Chile the only country in South and Central America free from malaria, and its potential for recurrence in malaria-free zones, the emergence of populations of parasites in Central and South America, Asia and Africa that are resistant to the major available antimalarial agents, and a decrease in vector control programs emphasize the need for continued mass screening of candidate antimalarial compounds.

A total of 289,191 three dose-level tests were performed from December 1, 1961 through September 30, 1983.

Table 1 summarizes the compounds tested and the number of mice used from December 1, 1961 through September 30, 1983.

The test system designed specifically for this operation is based on blood-induced Plasmodium berghei malaria infections in mice. It is a relatively simple and fast procedure. Assessment of antimalarial effect and host toxicity are reproducible and reliable.

All compounds evaluated were obtained from the Division of Experimental Therapeutics at the Walter Reed Army Institute of Research and included: 1) Compounds structurally related to chemicals of known value as antimalarial agents; 2) Compounds structurally unrelated to compounds known to have antimalarial activity; 3) Structural analogues of compounds found active in our test system and representing several novel chemical groups; 4) Compounds known to have activity against other infectious disease agents.

Our own breeding colony of ICR/HA Swiss mice has supplied all the animals used in our tests.

Drug activity was assessed by comparing the maximum survival time of treated malaria-infected animals to the survival time of untreated malaria-infected control mice.

Using five and six week old mice and a standard inoculum of *P. berghei*, it has been possible to produce a consistent disease fatal to 100% of untreated animals within six to seven days.

An established disease is less responsive to treatment than a disease in the early stages of development, therefore treatment was deliberately withheld until a moderately high degree of parasitemia was evident. Test compounds were administered subcutaneously in a single dose on the third day post-infection, at which time a 10-15% parasitemia had developed. A similar procedure was followed for the oral administration of selected active compounds.

A compound can only be classified as "active" if it suppresses the disease and produces an unquestionably significant increase, 100% or more, in the life span of the treated animals over that of the untreated infected controls. A compound is considered to be "curative" if the treated animals remain alive for 60 days after infection with *P. berghei*. Compounds not meeting the above requirement are considered inactive.

The severity of the challenge set up in our test system enhances the reliability of our evaluation and the antimalarial potential of the compounds selected for intensive preclinical studies.

## METHODS

### ANIMAL HOST

The total supply of animals needed to screen candidate compounds was obtained from our breeding colony of ICR/HA Swiss mice (Mus musculus). Test animals weighed from 18-20 grams. Weight variations in any given experimental or control group were carefully limited to within two to three grams. In any given test all animals were approximately the same age.

Animals on test were housed in metal-topped plastic cages, fed a standard laboratory diet and given water ad libitum. Once the infected mice had been administered the drug, they were placed in a room maintained at 28.8°C ( $\pm 2^\circ\text{C}$ ), and a relative humidity of 66%.

### TEST PROCEDURE

Test animals received an intraperitoneal injection of approximately  $5.98 \times 10^5$  parasitized erythrocytes drawn from donor mice infected four days earlier with P. berghei. The donor strain was maintained by passage every four days in separate groups of mice inoculated with 0.2 cc of a 1:435 dilution of heparinized heart blood.

To check factors such as changes in the infectivity of our P. berghei strain or in the susceptibility of the host, one group of mice, which served as the negative control, was infected but not treated. To determine the effect that a drug exerted upon a malaria infection, two parameters were measured; the first was an increase in survival time; the second concerned curative action. For comparative purposes, one standard compound, pyrimethamine, was administered at one level (120 mg/kg) to a group of 10 mice. Pyrimethamine served as a positive control, producing definite increases in survival time and curative effects. Another function of the positive control involved monitoring three procedures: The drug weighing, the preparation of drug solutions and suspensions, and the administration of drugs.

## DRUG ADMINISTRATION

Test compounds were dissolved or suspended in peanut oil before they were administered subcutaneously. Compounds to be administered orally were mixed in an aqueous solution of 0.5% hydroxyethylcellulose-0.1% Tween-80.

Treatment consisted of a single dose given subcutaneously or orally three days post-infection. At the time of treatment, a 10-15% parasitemia had developed. Although the disease was well established, it had not yet caused sufficient debility to affect an evaluation of the test compound's toxicity.

Deaths that occur before the 6th day, when untreated infected controls begin to die, were regarded as the result of a compound's toxic effects and not as the result of action by the infecting parasite.

Each compound was initially administered in three graded doses, diluted four-fold, to groups of five mice per dose level. The top dose was 640, 320, or 160 mg/kg, of body weight depending upon the amount of compound available for testing. Active compounds were subsequently tested at six or nine dose levels, diluted two-fold from the highest dose. Successive six-level tests were performed at respectively lower doses until the lower limit of activity was reached, thus establishing a complete dose-response picture for that compound in a rodent system.

A drug that was toxic for the host at each of the three levels initially tested was retested at six dose levels diluted two-fold from the lowest toxic dose.

## DRUG ACTIVITY

Acceptance of a drug as being sufficiently active for detailed studies was predicated on the margin between the maximum tolerated dose (MTD) and the minimum effective dose (MED) producing a significant effect. An MTD is defined as the highest dose up to 640 mg/kg causing no more than one of five animals to die from drug toxicity. The MED is defined as the minimum dose increasing the life span of treated animals by 100% over the life span of untreated controls.

Clearly inactive compounds were rejected after one test, borderline compounds were characterized by a dose-response curve, which established the spread between the MTD and the lower limit of activity by a determination of drug activity in the dose-level dilution tests. The total number of compounds and mice used from December 1, 1961 to September 30, 1983 is summarized in Table I. Table II depicts the number of active compounds during this time period.

# PLASMODIUM BERGHEI MALARIA IN MICE

TABLE I

## Summary of Screening Levels

December 1, 1961 - September 30, 1983

Time Period	Number of Compounds Tested	Number of Mice
October 1, 1982 - September 30, 1983	3,026	46,895
October 1, 1981 - September 30, 1982	3,020	45,900
October 1, 1980 - September 30, 1981	2,998	45,967
October 1, 1979 - September 30, 1980	4,826	74,040
October 1, 1978 - September 30, 1979	6,175	86,415
October 1, 1977 - September 30, 1978	5,375	82,690
June, 1976 - September, 1977	7,114	123,085
June, 1975 - May, 1976	9,916	155,585
June, 1974 - May, 1975	10,604	168,725
June, 1973 - May, 1974	11,035	168,664
June, 1972 - May, 1973	14,276	231,450
June, 1971 - May, 1972	14,874	262,245
June, 1970 - May, 1971	18,108	322,140
June, 1969 - May, 1970	22,376	411,270
June, 1968 - May, 1969	38,150	603,225
June, 1967 - May, 1968	40,465	636,525
June, 1966 - May, 1967	34,093	531,200
June, 1965 - May, 1966	22,731	350,449
June, 1964 - May, 1965	13,114	215,715
December, 1961 - May, 1964	6,915	250,000
TOTAL	289,191	4,812,185

PLASMODIUM BERGHEI MALARIA IN MICE

TABLE II

Summary of Active Compounds

June 1, 1970 - September 30, 1983

Time Period	Number of Compounds Tested	Number of Active Compounds
October 1, 1982 - September 30, 1983	3,026	335
October 1, 1981 - September 30, 1982	3,020	574
October 1, 1980 - September 30, 1981	2,998	359
October 1, 1979 - September 30, 1980	4,826	581
October 1, 1978 - September 30, 1979	6,175	969
October 1, 1977 - September 30, 1978	5,375	1,261
June 1, 1976 - September 30, 1977	7,114	1,124
June 1, 1975 - May 31, 1976	9,916	351
June 1, 1974 - May 31, 1975	10,604	616
June 1, 1973 - May 31, 1974	11,035	394
June 1, 1972 - May 31, 1973	14,276	771
June 1, 1971 - May 31, 1972	14,874	593
June 1, 1970 - May 31, 1971	18,108	805
	-----	-----
TOTAL	111,347	8,733

## SECONDARY ANTIMALARIAL SCREENING SYSTEMS

Current prospects for the control of human malaria have been complicated by the occurrence of drug-resistant parasites to all drugs available today to combat P. falciparum. Such resistance falls into the following categories:

1. Resistance to antifolate drugs (pyrimethamine, chloroguanine, etc.).
2. Resistance to 4-aminoquinolines, quinoline-carbinols and acridines (chloroquine, amodiaquine, atebine, quinine, etc.).
3. A combination of 1 and 2 which is referred to as multiple resistance.
4. Resistance to Fansidar (pyrimethamine plus sulfadoxine).

Collectively, the several types of resistance impair the effectiveness of all major suppressive drugs. Parasites of P. falciparum in Southeast Asia have been found to be resistant to mefloquine, a new quinoline methanol drug. Hence, a great need exists for alternative drugs as well as new combinations of drugs.

New candidate compounds are emerging from a primary blood schizonticidal screening program, and it is particularly important to determine quite early which of the new candidates are likely to be useful against the various types of drug-resistant malaria. Experience has indicated that plasmodia of animals can be used for this purpose.

The specific aims for this test system are to conduct a sequential battery of chemotherapeutic studies in P. berghei-infected mice on active compounds proprietarily discreet or non-discreet emerging from the Department of Defense sponsored screening programs in order to determine which substances are worthy of further consideration as potential agents for dealing with drug-resistant malaria.

The techniques used in this secondary drug-testing program fall into two categories:

1. Studies designed to determine if a new agent was likely to be useful against the various types of drug-resistant malaria.
2. General chemotherapeutic characterization of selected new agents to suggest optimal methods of use.

## METHODS

### ANIMAL HOSTS AND PARASITES

The testing was done with P. berghei in outbred ICR/HA female Swiss mice (Mus musculus) weighing 20-25 grams. Briefly, this testing entails procedures for the direct assessment of the effects of drugs on the parasitemia. Various tolerance observations were also recorded which served as guides indicating the usefulness of the new test agents as drugs for the treatment of malaria.

More specifically, activities included the elucidation of a possible mode of action of agents by testing them in parallel against drug-sensitive P. berghei (KBG-173) and various drug-resistant parasite lines which were chloroquine-resistant, cycloguanil-resistant, dapsone-resistant, mefloquine-resistant, pyrimethamine-resistant or quinine-resistant.

### TEST DESIGN

When a new compound was obtained it was subjected to a battery of testing procedures, the extent of which depended on its degree of activity in suppressing murine malaria infections. The first test procedure was a 6-day suppressive test against the drug-sensitive P-line.

If the compound was active against the P-line, then a 6-day test against one or more drug-resistant lines followed. In this basic 6-day suppressive test, mice were divided into groups of seven and inoculated with parasites intraperitoneally. Drugs were administered twice a day, usually orally, in a volume of 10 ml/kg on the third, fourth and fifth days after inoculation of parasites. All drugs were mixed in aqueous 0.5% hydroxyethylcellulose-0.1% Tween-80 and ultrasonicated when necessary. Drug doses were prepared using 100% of the free base of each drug. One group of ten infected mice received the vehicle alone and served as a negative control group. The blood films and final group weights were taken on the sixth day after inoculation of parasites. Microscopic examination of Giemsa-stained blood smears was made to determine the percentage of cells parasitized, percent suppression of parasitemia, and significance values for the suppression of parasitemias. Significance values were based on a calculation of the percent suppression of parasitemia which was determined by comparing the parasitemia of each treated mouse with the mean parasitemia of the negative control animals. Drug tolerance was reflected by the percent weight change and the proportion of mice that survive treatment. Toxicity was attributed to drug action when a 14% or greater weight change occurred or when one or more mice died before the blood smears were taken.

#### REGULAR P-LINE TESTING

Each new drug was tested first against the drug-sensitive P-line, usually via both oral and subcutaneous routes of administration. The drug dosages for the first test were normally 64, 16, 4 and 1 mg/kg/day for the three days. If more than 90% suppression of the parasitemia ( $SD_{90}$ ) was obtained with the lower dose of 1 mg/kg/day, then testing at lower doses was performed. Chloroquine was tested as a reference against the P-line at levels of 2, 3, and 4 mg/kg/day. A quinine index (QI) was calculated by comparing the  $SD_{90}$  value of the new compound to that of chloroquine:

$$Q = \frac{\text{SD}_{90} \text{ of chloroquine}}{\text{SD}_{90} \text{ of new compound}} \times 30^*$$

\* Conversion factor (chloroquine is 30 times more active than quinine).

### DRUG-RESISTANT LINES

Compounds that suppressed the P-line parasitemia by at least 90% with 64 mg/kg or less were subjected to testing against one or more of the six drug-resistant lines. These lines included a chloroquine-resistant (C-line), a cycloguanil-resistant (T-line), a dapsone-resistant (S-line), a mefloquine-resistant (A-line), pyrimethamine-resistant (M-line) and a quinine-resistant line (Q-line). The amount of testing against the resistant lines depended upon the structure of each compound as it related to the structure of known antimalarials. A maximum dose of 256 mg/kg/day was administered orally along with doses of 64, 16 and 4 mg/kg/day.

### ESTIMATES OF POTENCY AND CROSS RESISTANCE

Doses required for a given degree of effect, such as 90% suppression or  $\text{SD}_{90}$ 's were used to delineate the degree of cross resistance.

### SPECIAL STUDIES

#### 1) Stability of drug resistance without drug pressure.

The six drug-resistant lines of malaria; A-line resistant to mefloquine, C-line resistant to chloroquine, M-line resistant to pyrimethamine, Q-line resistant to quinine, S-line resistant to dapsone, and the T-line resistant to cycloguanil were removed from drug pressure and followed to see how long resistance was retained.

2) Determination of the development of resistance to mefloquine when sulfadoxine and pyrimethamine were co-administered.

A fixed (20:1) combination of sulfadoxine: pyrimethamine (FANSIDAR<sup>R</sup>) in a single oral dose administered with mefloquine has been advocated as a means of delaying the development of resistance to mefloquine. The antimalarial activity of these three drugs singly and in combination, was, therefore, evaluated in mice infected with Plasmodium yoelli. Seven groups of five mice each were inoculated intraperitoneally with  $1.0 \times 10^7$  parasitized red blood cells. Three, four and five days after infection, mice received a single daily oral dose of drug or drug combination according to body weight. Seven days after infection, blood films were made to determine the degree of infection. The group of mice yielding a 5% parasitemia were bled and used to infect the next group of uninfected mice. Sulfadoxine-pyrimethamine concentration was kept at 0.0125 mg/kg - 0.003 mg/kg. Mefloquine concentration began at 4 mg/kg and increased as resistance developed until resistance to 256 mg/kg was obtained.

3) Development of mefloquine resistance in a standardized method.

A series of experiments were performed to develop a standard procedure for induction of mefloquine resistance. The drug pressure was increased as rapidly as possible in a uniform schedule. The procedure is as follows.

Parasite: Plasmodium yoelli yoelli 17X

Inoculum:  $1 \times 10^7$  parasitized RBC i.p. in 0.1 ml volume unwashed whole blood diluted in saline.

Mefloquine administration: Orally in HEC/Tween suspension, 0.01 ml/gm body weight via metal esophageal cannula. Mefloquine administration once daily on days 3, 4, and 5 post inoculation.

<u>Group</u>	<u>Fold-Increase in Mefloquine</u>
A	8
B	4
C	2
D	1.5
E	1.25
F	1.0
G	0.5

Parasitemia: On day 7 post inoculation blood films were taken and parasitemias determined.

Passage of parasites: Passage was made from the single mouse having a parasitemia >5% in the highest dosage group. That dose would then be 1-fold in the next passage. This was done until the parasites were resistant to 256 mg/kg of mefloquine.

## RESULTS

### 6 Day Test

The results for the regular 6-day suppressive test against the drug-sensitive and drug-resistant lines are summarized in Table III.

### Stability of drug-resistant lines.

The chloroquine and pyrimethamine-resistant lines lost resistance between 9-12 months. The remaining lines have retained their respective resistance for 36 months.

### Study to examine if the development of mefloquine resistance could be slowed by co-administration of sulfadoxine and pyrimethamine.

Development of full mefloquine resistance, when administered alone, occurred within 6 weeks. Development of full mefloquine resistance, when administered with FANSIDAR, occurred within 6 to 9 weeks. There appears to be no advantage of combining FANSIDAR with mefloquine to slow the development of mefloquine-resistant parasites.

### Development of mefloquine resistance in a standardized method.

Parasites resistant to 256 mg/kg of mefloquine were developed in 4 to 8 passages using the standardized method.

TABLE III

Summary of data for regular 6-day testing with drug-sensitive  
and drug-resistant parasites.

WR No. Bottle No.	EXP. No.	SD (mg/kg/day) <sup>1</sup>								
		2 P	3 A	4 C	5 M	6 T	7 S	8 X	9 Z	
448 AG 28874	347						>256	P0	>32	
1544 AR 20613	341 347			>128			2.6	P0 P0	>45 0	
42313 BJ 92609	338 339 339 340 340 341	2.8 3.7 5.9	>16			>16		P0 P0 SC P0 P0 P0	>5   >2 >2 >2	
142490 BG 59453	338		>256					P0	>54	

TABLE III (Cont.)

WR No. Bottle No.	EXP. No.	SD (mg/kg/day)								
		1	2	3	4	5	6	7	8	9
		P	A	C	M	T	S	X	Z	
169626 BK 09350	340 340	14				33		P0 P0	2.3 2	
171669 BB 43914	338 347		>256				2.3	P0 P0	>150 0	
178460 BK 21070	338 339 339 347	0.43 1.4	>256					P0 P0 SC P0	>595 5.8	
226768 BG 47364	338		33					P0	0	
233599 BH 13470	338		>4					P0	<2	
233600 BH 50624	338		>4					P0	<2	

TABLE III (Cont.)

WR No. Bottle No.	EXP. No.	SD <sup>1</sup> (mg/kg/day)									
		2	3	4	5	6	7	8	9		
		P	A	C	M	T	S	X	Z		
250050 BK 16793	341			>256				P0	>26		
250051 BK 16800	341			>256				P0	>12		
250052 BK16784	341 347			>256			28	P0 P0	>27 0		

1 = Amount of drug to suppress 90% of the parasites.

2 P = drug-sensitive line.

3 A = mefloquine-resistant line.

4 C = chloroquine-resistant line.

5 M = pyrimethamine-resistant line.

6 T = cycloguanil-resistant line.

7 S = dapson-resistant line.

8 R = P0 = oral route of drug. SC = subcutaneous route of drug.  
X

SD<sub>90</sub> drug-resistant line

9 Z = Degree of cross resistance =  
SD<sub>90</sub> drug-sensitive line

TABLE IV

Comparison of suppressive activities of drug-resistant parasites away from drug pressure with drug-resistant parasites maintained on drug pressure.

Month away from drug pressure									
		21		24		27		36	
		Mefloquine		Mefloquine		Mefloquine		Mefloquine	
		2		3		4		*	
		P	A	P	A	P	A	P	A
1	SD	3.4	>256	1.4	>256	3.9	>256	2.4	>256
	90								
		Quinine		Quinine		Quinine		Quinine	
		*		*		*		*	
SD		P	Q	P	Q	P	Q	P	Q
	90	73.0	205	74.0	155	51.0	230	64	230
		>256		240		>256		>256	
		Dapsone		Dapsone		Dapsone		Dapsone	
		*		*		*		*	
SD		P	S	P	S	P	S	P	S
	90	0.31	>256	0.5	>256	0.3	>256	0.3	>256
		>256		>256		>256		>256	
		Cycloguanil		Cycloguanil		Cycloguanil		Cycloguanil	
		*		*		*		*	
SD		P	T	P	T	P	T	P	T
	90	2.2	250	4.0	>256	1.2	>256	2.2	>256
		>256		>256		>256		>256	

2 P = Drug sensitive line.

\* \* \* \*

3 A, Q, S, T = Drug-resistant lines removed from drug pressure.

4 A, Q, S, T = Drug-resistant lines maintained on drug pressure.

Chloroquine-resistant parasites lost resistance between 9-12 months.

Pyrimethamine-resistant parasites lost resistance between 9-12 months.

TABLE V

A comparison of degrees of resistance at SD<sup>90</sup> of drug-resistant parasites away from drug pressure for 21, 24, 27 and 36 months with drug-resistant parasites maintained on drug pressure.

Months away from drug pressure				
21	24	27	36	
Mefloquine	Mefloquine	Mefloquine	Mefloquine	
* A >75	* A >182	* A >65	* A >106	A >106
Quinine	Quinine	Quinine	Quinine	
* Q 2.8	* Q 2.1	* Q 4.5	* Q 3.6	Q >4
Dapsone	Dapsone	Dapsone	Dapsone	
* S >825	* S >512	* S >981	* S >853	S >853
Cycloguanil	Cycloguanil	Cycloguanil	Cycloguanil	
* T 113	* T >64	* T >213	* T >116	T >116

1. A, Q, S, T = Drug-resistant lines removed from drug pressure.

2. A, Q, S, T = Drug-resistant lines maintained on drug pressure.

## SPOROZOITE INDUCED ANTIMALARIAL TEST IN MICE

There is no causal prophylactic drug available today for people to take in order to stop the development of exoerythrocytic stages of the four species of human malaria.

Primaquine is the only drug currently used today for radical curative activity in humans (elimination of persisting exoerythrocytic schizonts and or hypnozoites of Plasmodium vivax and Plasmodium ovale). This 8-aminoquinoline has two major limitations; it has a poor therapeutic index, and it causes hemolytic anemia in persons with a deficiency in the enzyme glucose-6-phosphate dehydrogenase. An additional liability concerns its ability to cause methaemoglobinaemia in individuals heterozygous for NADH-methaemoglobin reductase deficiency when taking primaquine. New active 8-aminoquinolines as well as other groups of chemicals exhibiting causal prophylactic and radical curative activity are needed to combat malaria in the world today.

This test system is intended to serve as a primary screening procedure for compounds submitted by the Division of Experimental Therapeutics at the Walter Reed Army Institute of Research.

In this test system mice received a subcutaneous injection of drug four hours prior to an intraperitoneal inoculation of sporozoites and survival was monitored for a 30 day period. A similar procedure was followed for the oral administration of selected active compounds. Mice alive after 30 days were considered cured.

## METHODS

### ANIMALS

Male or female outbred ICR/HA Swiss mice (Mus musculus), six to seven weeks old, weighing 16 to 17 grams were used as test animals. They were maintained in groups of five and had free access to feed and water.

Mice used as a source of gametocytes (donor mice) were eight weeks old and weighed 25 to 30 grams.

#### MOSQUITO COLONY

Anopheles stephensi were reared in an insectary maintained at 26.7°C ( $\pm 2^\circ\text{C}$ ) and 70% ( $\pm 2\%$ ) relative humidity with 14 hours of light and 10 hours of darkness. Larvae were fed a solution of 2.5% liver powder once a day. Emerged adults were fed a 10% glucose solution ad lib.

#### INFECTED MICE AS A SOURCE OF GAMETOCYTES

Donor mice to be used as a source of gametocytes were injected intraperitoneally with a dilution of infected heart blood from mice previously infected with sporozoites of Plasmodium yoelii yoelii 17X. These mice were used two to three days after inoculation with parasitized red blood cells. The gametocytes developed within 48 to 72 hours and produced a uniform infection in the mosquitoes.

#### INFECTION OF MOSQUITOES

Mosquitoes were placed in a room maintained at 21.1° C ( $\pm 2^\circ\text{C}$ ) and 70% ( $\pm 2\%$ ) relative humidity prior to receiving the infected blood meal. Donor mice harboring a 2 to 10% parasitemia were anesthetized with Nembutal and placed on top of the mosquito cages for one hour to allow the mosquitoes to feed on infected blood. A second infected blood meal was given the following day, and thereafter the mosquitoes were maintained on a 10% glucose solution. A single normal blood meal was given seven days after the first infected blood meal.

#### ISOLATION OF SPOROZOITES

Eighteen days after the first infected blood meal the mosquitoes were anesthetized with ether, vacuumed into plastic

bags kept immobilized on a cold table ( $-5^{\circ}$  to  $0^{\circ}\text{C}$ ). The females were separated from the males and placed into a cold glass mortar. The males were discarded. After approximately 500 females were collected, one ml each of saline and heat inactivated mouse plasma were added and the suspension was macerated with a glass pestle for three minutes. An additional 20 ml of saline and mouse plasma (1:1) were added to the suspension and filtered through nylon monofilament screening fabric with a mesh opening of 90 microns. This step removed large tissue fragments of the mosquitoes, yet allowed the sporozoites to freely pass through into the refined suspension. This filtered sporozoite suspension was further diluted to obtain a concentration of approximately  $2.5 \times 10^5$  sporozoites per 0.2 ml of inoculum.

#### ADMINISTRATION OF TEST COMPOUNDS

Each compound was ground with a mortar and pestle and then suspended in a quantity of 0.5% hydroxyethylcellulose-0.1% Tween-80 to obtain the desired drug dose. The percent free base of each compound was not determined. Four hours prior to the inoculation of sporozoites, compounds were administered either subcutaneously or orally at three graded doses diluted four-fold (160, 40 and 10 mg/kg). Groups of five mice per dose level were used. Subsequent tests used successively lower four-fold dilutions of test compound if mice were cured at 10 mg/kg until the lower limit of a compound's activity was reached.

Infected control mice (receiving sporozoites only) began to die due to malaria starting seven days after inoculation of sporozoites. Deaths that occurred prior to seven days in mice treated with test compounds were considered drug toxicity deaths. A drug that was toxic to the host at each of the three initial dose levels was retested at doses diluted four-fold from 10 mg/kg.

### INOCULATION OF MICE WITH SPOROZOITES

Mice were injected intraperitoneally with approximately  $2.5 \times 10^5$  sporozoites. Twenty of these mice were divided into two groups of ten each. One group received no drug and served as a negative control. The other group was treated with WR 181023 (125 mg/kg) and acted as a positive control. One additional control group of five infected mice was treated with chloroquine (100 mg/kg).

### DETERMINATION OF ANTIMALARIAL ACTIVITY

After the mice were inoculated with sporozoites they were placed in a room maintained at  $28.8^\circ\text{C}$  ( $\pm 2^\circ\text{C}$ ) and 66% ( $\pm 2\%$ ) relative humidity. Antimalarial activity was determined by monitoring daily mortality. Mice which were alive after 30 days were considered cured. A compound was considered active if at least two mice survived for 30 days at any dose level. Active compounds were retested at 160, 40, 10 both subcutaneously and orally.

## RESULTS

### CONTROLS

Mice inoculated with sporozoites but receiving no drug (negative control groups) all routinely died within 7 to 12 days, as did mice receiving chloroquine. Mice serving as positive controls receiving 125 mg/kg of WR 181023 (4-methyl-primaquine) usually survived for the duration of the experiment (30 days).

### COMPOUNDS TESTED AND DRUG ACTIVITY

For the period October 1, 1982 to September 30, 1983, 1571 three-level tests were performed. 38 compounds were active both orally and subcutaneously; 12 compounds were active only subcutaneously, while 10 were active only orally. 29 compounds

tested only subcutaneously were active. 1 compound was active when tested only orally. 1317 compounds were inactive when tested only subcutaneously. 45 compounds were found to be inactive both subcutaneously and orally while 14 compounds were inactive when tested only orally.

A SCREENING PROCEDURE FOR THE EVALUATION  
OF TRYPANOCIDAL ACTIVITY OF CANDIDATE COMPOUNDS  
IN TRYPANOSOMA RHODESIENSE INFECTED MICE

According to the World Health Organization, there is no adequate information on the prevalence of human African trypanosomiasis. The best estimates report that 35 million people at risk, and about 20,000 new cases are reported annually. African trypanosomiasis has a very high mortality rate and has considerable importance as a public health problem, especially in this age of increasing foreign travel.

No new trypanocidal drugs have been introduced since the synthesis of pentamidine in 1939. Four drugs are currently available for the treatment of human trypanosomiasis caused by Trypanosoma rhodesiense or Trypanosoma gambiense. Two of these drugs, suramin and pentamidine are used in the treatment of the blood parasite (trypomastigote), but lack efficacy in the treatment of central nervous system infections (trypomastigote). Melarsoprol and nitrofurazone are used for the treatment of amastigotes in the central nervous system.

Each of these four drugs have severe side effects resulting in poor therapeutic indices. The use of suramin may lead to nausea, vomiting, shock and loss of consciousness. It can also cause exfoliative dermatitis, albuminuria, hematuria and ultimately renal failure. Pentamidine use may lead to fatal hypertension, hypoglycemia, diabetes and renal dysfunction. Administration of melarsoprol may lead to lethal encephalopathy in 10 to 15 percent of cases. Nitrofurazone can cause severe polyneuropathy and degeneration of the seminiferous tubules. This drug is also associated with causing hemolytic anemia in glucose-6-phosphate dehydrogenase deficient patients.

Compounding the problem of low therapeutic indices is the problem of trypanosomal drug resistance. Human trypanosome strains are commonly resistant to at least one chemotherapeutic agent. With some patients, their infection is resistant to two or more antitrypanosomal drugs.

Therefore, there is a definite need to develop and test compounds that are potentially active against resistant strains of T. rhodesiense and that are less toxic than the existing drugs. Further testing also needs to be done using different routes of administration and combinations of two or more drugs.

The test system described herein was developed specifically to evaluate the trypanosomal activity of large numbers of candidate compounds. Based on blood induced T. rhodesiense infections in mice, it acts as a primary screen or as a secondary screen and/or confirmatory test. This test gives precise quantitative evaluation of chemical compounds that demonstrate potentially useful therapeutic and/or prophylactic activity in T. rhodesiense infections. Consequently, it is also a helpful guideline in the synthesis of new related active agents.

All candidate compounds are obtained from the chemical inventory of the Division of Experimental Therapeutics at the Walter Reed Institute of Research and include:

1. Chemicals structurally related to compounds of known value in the treatment or prevention of T. rhodesiense infections;
2. Chemicals structurally unrelated to compounds of known value in the treatment or prevention of T. rhodesiense infections;
3. Structural analogues of compounds that have demonstrated activity in our screening procedure and represent novel chemical groups;
4. Compounds known to have activity against other parasitic and bacterial infectious agent.

## METHODS

### ANIMAL HOST

ICR/HA Swiss mice (Mus musculus) used in this screening procedure weighed 28 to 30 grams with weight variation in any given experimental or control group carefully limited to three grams. Male and female mice approximately the same age were used.

Animals were housed in metal-topped plastic cages, fed a standard laboratory diet and given water ad libitum. After drug treatment, mice were kept in a room maintained at a temperature of 28.8°C ( $\pm 2^\circ\text{C}$ ) with a relative humidity of 66% ( $\pm 2\%$ ).

### TEST PROCEDURE

Test animals received an intraperitoneal injection of 0.2 cc of a  $1.5 \times 10^4$  dilution of heparinized heart blood drawn from a donor mouse infected three days earlier (approximately  $1.3 \times 10 - 1.7 \times 10^4$  trypomastigotes).

The donor line was maintained by three-day blood passes; each animal received 0.1 cc of a  $1:5.0 \times 10^2$  dilution of heparinized heart blood drawn from a mouse harboring a 3 day infection.

One group of infected, untreated mice was included as a negative control to check both the infectivity of the I. rhodesiense (CT-Wellcome strain) and the susceptibility of the murine host. In order to determine the effect a drug exerted on a trypanosome infection, two parameters were measured; 1) the increase in mouse survival time and 2) drug curative action. For comparative purposes, two standard antitrypanosomal compounds, stilbamidine isethionate and 2-hydroxystilbamidine isethionate, are administered subcutaneously at one dose (26.5 mg/kg) to separate groups of ten mice. The same positive controls were administered orally at 53 mg/kg when compounds are tested orally. These two diamidines served as positive controls, producing definite increase in survival time and curative effects.

## DRUG ADMINISTRATION

Test compounds were dissolved or suspended in peanut oil before they were administered subcutaneously. Compounds to be administered orally were mixed in an aqueous solution of 0.5% hydroxyethylcellulose 0.1% Tween-80.

Treatment consisted of a single dose, given subcutaneously or orally, two to three hours after the injection of parasites. Deaths that occurred before the fourth day, when untreated infected controls begin to die, were regarded as a result of toxic action by the drug, not the lethal effects of the parasites.

Each compound was initially administered in three graded doses diluted four-fold to groups of five mice per dose level. The top dose was either 424, 212, or 106 mg/kg, depending on the amount of compound available for testing. Active compounds were subsequently tested at six dose levels, diluted two-fold from the highest dose. If necessary, successive six-level tests were performed at respectively lower doses until the lower limit of activity was reached.

A drug that was toxic for the host at each of the three levels initially tested was retested at six dose levels diluted two-fold from the lowest toxic dose.

## DRUG ACTIVITY

Acceptance of a drug as being sufficiently active for detailed studies was predicated on the margin between the maximum tolerated dose (MTD) and the minimum effective dose (MED) producing a significant effect. A MTD is defined as the highest dose up to 424 mg/kg causing no more than one of five animals to die from drug toxicity. The MED is defined as the minimum dose increasing the life span of treated animals by 100% over the life span of untreated infected controls.

Clearly inactive compounds were rejected after one test and border-line compounds after two tests. Active compounds are characterized by dose-response curves, which establish the spread between the MTD and the lower limit of activity by a determination of drug activity in the dose-level dilution tests. Treated animals alive at the end of 30 days were considered cured.

## RESULTS

### CONTROLS

Mice inoculated with trypomastigotes but receiving no drug (negative control group) all routinely died within 4 to 5 days. Mice serving as positive controls, receiving 26.5 mg/kg of stilbamidine, or 26.5 mg/kg of 2-hydroxystilbamidine, usually survived for the duration of the experiment (30 days).

### NEW COMPOUNDS

Data for all the new compounds is summarized in Table VI.

TABLE VI

Summary of *Trypanosoma rhodesiense* drug screen test

Report Period	Number of three- level tests	Number of mice utilized	Number of compounds tested	Total number of active compounds	Number of compounds active by route of administration			
					Oral only	S.C. only	1 Oral and S.C.	2 I.P. only
10/1/82-9/30/83	2,069	32,835	1,788	82	0	75	7	0
10/1/81-9/30/82	1,994	32,405	1,960	67	3	60	2	2
10/1/80-9/30/81	2,043	32,520	1,222	62	6	50	6	0
10/1/79-9/30/80	4,780	60,110	3,462	88	3	78	7	0
10/1/78-9/30/79	3,158	49,708	2,783	125	7	116	2	0
10/1/77-9/30/78	4,025	64,600	3,032	77	9	54	14	0
6/1/76-9/30/77	4,235	73,280	4,235	396	17	270	109	0
6/1/75-5/31/76	1,653	30,290	1,653	257	59	198		
6/1/74-5/31/75	1,826	33,850	1,826	298	73	225		
6/1/73-5/31/74	1,581	25,360	1,581	185	93	92		
8/1/72-5/31/73	3,030	51,405	3,030	68				
TOTAL	30,394	486,363	26,572	1,705	270	1,218	147	2

1  
S.C. = Subcutaneous

2  
I.P. = Intraperitoneal

## DRUG-RESISTANT TRYPANOSOME TEST

The resistance of *T. rhodesiense* to selected antitrypanosomal compounds can be induced by repeated drug pressure in an in vivo test system. This is achieved by infecting mice with a standard inoculum of parasites, administering the test compound at a dose just below the curative level, and passing parasites from these animals to a new set of mice when the parasitemia rises to a desirable level. Passes are made every three to four days with drug doses being increased as resistance develops at each dose level.

This type of study can establish the rate at which *T. rhodesiense* acquires resistance in mice to selected compounds. Degrees of cross resistance to trypanosomicidal compounds found to be active against the drug-sensitive line in primary screening tests may also be determined.

Lines of trypanosomes have been developed which are completely resistant to the following compounds.

<u>RESISTANT LINE</u>	<u>HIGHEST DOSE ACHIEVED</u>
Pentamidine	212.0 mg/kg
Melarsoprol (Mel-B)	424.0 mg/kg
Suramin	543.0 mg/kg

## METHODS

### ANIMAL HOST

ICR/HA Swiss mice (*Mus musculus*) used in this screening procedure weighed 24 to 26 grams with weight variations in any given experimental or control group carefully limited to three grams. Both male and female mice were used and were approximately the same age.

Animals were housed in metal-topped plastic cages, fed a standard laboratory diet and given water ad libitum. After drug treatment, mice were kept in a room maintained at a temperature of 28.8°C ( $\pm 2^\circ\text{C}$ ) with a relative humidity of 66% ( $\pm 2\%$ ).

#### DEVELOPMENT AND MAINTENANCE OF DRUG-RESISTANT LINES

On day zero, fifteen male or female mice were divided into three groups of five animals. All animals were initially inoculated intraperitoneally with drug-sensitive T. rhodesiense (Wellcome CT-strain) trypomastigotes in saline diluted blood (1:500) drawn from a previously infected donor mouse. Group I served as a negative control, receiving no drug. Group II received drug either orally or subcutaneously on day 0 and day 1. Group III was given the same dose of drug by the same route on day 0 only. On day 3 or 5, 15 new mice were infected with saline-diluted blood (1:500) from Group II. The pass was made from Group III if Group II animals demonstrated no parasites upon blood examination. These newly infected mice were similarly divided into three groups and given the same drug regimen as that just described. Passes were thus made every three to four days from the most recently infected and treated groups of animals. Drug doses were increased as resistance developed.

Once complete resistance to the highest tolerated dose of the compound was reached, the line was passed twice each week using two groups of five mice each. Group I mice received no drug and served as a negative control. Group II mice received a low dose of drug to maintain drug pressure and served as donor mice for the next passage.

#### TEST PROCEDURE - INOCULATION OF PARASITES

Giemsa-stained blood smears from donor mice infected three days earlier with T. rhodesiense trypomastigotes were microscopically examined to determine parasitemias (i.e., number of trypomastigotes in a field of 100 erythrocytes). One set of test animals was infected with the drug-sensitive line of parasites and received an intraperitoneal injection of 0.2 cc of

a  $1:15 \times 10^4$  dilution of heparinized heart blood drawn from a donor mouse harboring a parasitemia of 30-35% (approx.  $1.3 - 1.7 \times 10^4$  trypomastigotes). Other sets of mice were similarly infected with each drug-resistant line to be tested. Blood dilutions were made such that all mice infected with the resistant lines received approximately the same number of trypomastigotes as mice infected with the drug-sensitive line.

Groups of ten mice infected with the sensitive line and with each resistant line served as negative control, received no drug.

#### DRUG ADMINISTRATION

Test compounds were dissolved or suspended in either peanut oil for subcutaneous administration or 0.5% hydroxyethylcellulose -0.1%-Tween-80 for oral administration. Compounds were given one hour following challenge with trypomastigotes.

Compound doses were diluted two or four-fold from a level that had been projected to be fully curative. Five mice were used for each dose level.

#### COMPOUND ACTIVITY

Mortality was used as an index of drug activity. Untreated infected (negative control) mice routinely die on day 4 or 5 after inoculation of parasites. Increases in life span relative to that of the negative controls at each dose level were recorded. Curative activity was used in assessing the level of resistance of selected compounds. Mice surviving for 30 days were considered cured.

### RESULTS

#### EXPERIMENTAL DATA

Data for various active antitrypanosomal compounds is presented in Table VII to show their degree of cross resistance

with a line resistant to Mel-B, one resistant to suramin, and one resistant to pentamidine.

The loss of drug resistance without drug pressure has been studied. Mel-B resistance and pentamidine resistance have been lost at 10 and 15 weeks respectively. Suramin resistance has been stable for over one year.

TABLE VII

a

Degrees of resistance of the melarsoprol-resistant and suramin-resistant lines of *Trypanosoma rhodesiense* to eleven antitrypanosomal compounds.

Compound	Compound		Degree of Resistance	
	WR. No.	Bottle No.	Melarsoprol-Resistant Line	Suramin-Resistant Line
<b>Arsenotriazine</b>				
Mel-Ni	247229	BJ 45271	>151	
Mel-Ni	249119	BJ 45271	>1927	3.0
Mel-B	216692	BG 80510	>365	
Mel-B	216692	BG 80510	>353	1.0
	214133	BJ 92369	>1630	2.8
<b>Acridine</b>				
Acridflavin	65585	AP 76740	>37	
<b>Diamidine</b>				
Stilbamidine	30457	AH 55296	>55	3.0
Pentamidine	4931	BJ 92341	>132	1.4
Pentamidine	4931	BG 11391	>1060	
	249987	BK 15607	>132	1.6
<b>Diamidineoxine</b>		BK 52084	78	2.4
<b>Guanidine</b>				
	250372	BK 46219	>883	23.3
		BK 47994	>1247	58.2
		BK 51925	>1	>1.1
		BK 50277	>10	>10.4
<b>Phenanthridine</b>				
Ethidium bromide	141377	BE 19397	2.8	
<b>Sulfonated naphthalene</b>				
Suramin	17349	BH 58595	1.9	
<b>Thiazole</b>		BG 91175	1.0	

<sup>a</sup> Degree of resistance is determined by comparing CD<sub>50</sub> values for each compound when tested against the drug-sensitive and drug-resistant line (Degree of resistance =  $\frac{\text{CD}_{50} \text{ of resistant line}}{\text{CD}_{50} \text{ of sensitive line}}$ ).

## THE CHEMOTHERAPY OF EXPERIMENTAL CHAGAS' DISEASE

Infection by the haemoflagellate Trypanosoma cruzi, the etiologic agent of Chagas' disease, presents a devastating public health problem for millions of people in Central and South America. Reduviid bugs transmit T. cruzi to man through their feces as they bite man causing a disease characterized by an acute phase and or a subsequent chronic phase. In addition to the cultural, social and economic factors that makes Chagas' disease particularly difficult to manage, the problem is compounded by this protozoan's cellular invasiveness and its ability to cause immunopathology. The organism also lives in over 100 mammalian species. No satisfactory course of drug therapy has been found that will treat all stages of the infection while remaining non-toxic to humans. A new compound is also needed to kill trypomastigotes in blood used during blood transfusions.

The test system described herein serves to evaluate prospective chemotherapeutic agents against experimental Chagas' disease by obtaining the following information:

1) A compound's antiparasitic activity measured by the mean percent increase in survival time compared with infected untreated controls. Mice were monitored for 40 days.

2) A compound's toxicity. Death of treated mice before negative controls begin to die is attributed to compound toxicity.

3) An estimate of the compound's therapeutic index.

### METHODS

#### ANIMALS HOST

Male ICR/HA Swiss randomly bred mice six to seven weeks old weighing 18-21 grams were used.

Animals were housed in metal-topped plastic cages fed a standard laboratory diet and were given water ad. libitum.

## TEST PROCEDURE

On day zero, mice were inoculated intraperitoneally with approximately  $1.3 \times 10^5$  trypomastigotes in blood drawn from donor mice infected one week previously with *T. cruzi* trypomastigotes (Y-strain). Within 30 minutes following challenge, mice were given a single subcutaneous injection of the test compound mixed in peanut oil. Each compound was initially tested at three-dose levels, usually 640, 160 and 40 mg/kg unless known toxicity data suggested lowering the top dose. The end point in activity of each active compound was determined. Mortality was recorded daily for a period of 40 days after the challenge with parasites.

Infected negative controls received an injection of peanut oil. This group consistently died within 9-15 days after the intraperitoneal inoculation of parasites. A positive control drug was included in each experiment. The 5-nitrofurantoin, Lampit, known to have limited therapeutic value in treating patients with Chagas' disease, was used as a positive control. It has been found to delay the onset of mortality as well as allowing some mice to survive the 40 day period after being administered as a single subcutaneous dose of 640 mg/kg.

A classification system was used to assess the relative activity of prospective compounds by comparing the life span of treated animals to the longevity of negative controls. Schizotrypanocidal activity was divided into three categories; positive, minimal and negative. A positive compound was one producing at least a 50% increase in life span of mice over that of the negative controls. A minimal compound produced a 20 to 49.9% increase in longevity, and a compound producing less than 20% increase in life span is considered negative.

Active compounds prevent or delay acute mortality. The test system, as designed, does not assure that mice living past the 40 day observation period are cured. If complete elimination of the parasite is not attained during the acute stage of infection and the animal survives, a chronic stage follows.

## RESULTS

### CONTROL GROUPS

Mice infected but receiving no test compound (negative controls groups) routinely died between days 9 and 15 after injection of trypomastigotes.

Mice inoculated with trypomastigotes then treated with the 5-nitrofurantoin, at 640 mg/kg served as a positive control group. Some of these mice usually survived for the duration of the experiment, 40 days or had a prolonged survival time.

### EXPERIMENTAL DATA

A summary of compounds tested from 1979 to 1983 is presented in Table VIII.

TABLE VIII

Summary of *Trypanosoma cruzi* test results

Report Period	Number of three- level tests	Number of mice utilized	Number of compounds tested	Total Number of active compounds	Number of compounds active by route of administration		
					S.C. only	Oral only	S.C. and oral
10/1/82-9/30/83	126	3,740	120	3	3	0	0
10/1/81-9/30/82	105	2,455	93	3	3	0	0
10/1/80-9/30/81	187	4,205	162	16	14	1	1
10/1/79-9/30/80	230	5,290	212	21	20	0	1
4/4/79-9/30/79	114	2,710	111	7	7	0	0
	---	-----	---	--	--	-	-
TOTAL	762	18,400	698	50	47	1	2

## A C K N O W L E D G M E N T

The personnel at the Rane Laboratory participating in this Chemotherapy of Malaria project deserve a tremendous degree of credit for an excellent performance.

### CHEMOTHERAPY ASPECTS

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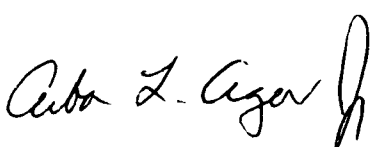
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